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Transcriptional regulation of the lipid transfer protein gene *LTP*3 in cotton fibers by a novel MYB protein☆

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Abstract

Two cotton fiber cDNAs (*GhMyb*7 and *GhMyb*9) and their corresponding genes, encoding R2R3-MYB proteins, have been isolated from the allotetraploid cotton (*Gossypium hirsutum* L. cv. DES119) and characterized. Genomic origin analysis reveals that *GhMyb*7 and 9 are alloallelic genes in the allotetraploid cotton (AD genome). *GhMyb*7 is derived from the A_2 subgenome, whereas *GhMyb*9 is from the D_5 subgenome. Northern blot analysis showed that *GhMyb*7/9 is expressed in flowers and fibers, and its expression in fibers is developmentally regulated. Auxin treatment increases transcript levels of *GhMyb*7/9 in fiber cells in an in vitro ovule culture system. The *Escherichia coli*-expressed GhMYB7 fusion protein binds to the promoter region (nt −614 to −580) of *Ltp*3, a cotton–fiber-specific gene, in an in vitro DNA–protein-binding assay, suggesting that GhMYB7/9 may play a role in the transcriptional regulation of the *Ltp*3 gene during fiber development. The overexpression of *GhMyb*7 in both transgenic tobacco and *Arabidopsis* plants causes a pleiotropic effect on plant development, including dwarf, abnormal leaf shape, and retarded root development. The late-flowering phenotype is observed in the 35S:*GhMyb*7 transgenic tobacco and *Arabidopsis* plants under long day (LD) condition. The transgenic *Arabidopsis* plants, however, show the absence of inflorescence under short day (SD) condition, suggesting that GhMYB7 overexpession might inhibit the GA biosynthesis or signaling pathway.

Keywords: Gossypium (cotton); R2R3-Myb; Transcriptional regulation; Ltp3; Auxin; Gibberellic acid

1. Introduction

MYB proteins are a group of DNA-binding proteins and serve as transcriptional factors to regulate the expression of downstream genes [1]. The typical MYB proteins contain three functional domains, including a basic DNA-binding domain (DBD), an acidic transactivation domain (TAD), and a negative regulatory domain (NRD) [1]. The DBD, located at the N-terminus of MYB proteins, is a structurally conserved domain, which consists of two or three imperfect repeats (R1, R2, and R3), and these repeats fold into a helix-turn-helix (HTH) structure to interact with DNA molecules [2]. The TAD and NRD located at the C-terminus of the proteins are variable within MYB proteins and are believed to be involved in protein–protein interactions [1].

Plants express a large family of R2R3-MYB proteins. A total of 125 *R2R3-Myb* genes are identified in *Arabidopsis thaliana* [3,4], over 80 found in maize [5], and at least 40 in *Petunia hybrida* [6]. Plant R2R3-MYB transcription factors have been shown to participate in many different pathways of plant growth and development, including secondary metabolism, cell differentiation, cell cycle, signal transduction, environmental response, and disease resistance [7]. An *Arabidopsis Myb* gene, *GLABROUS* 1 (*GL*1), has been identified as an essential gene for the initiation of leaf trichomes [8]. The *MIXTA* gene from *Antirrhinum majus*, encoding a MYB protein, has been reported to mediate the

☆ The sequences of *GhMyb*7 and *GhMyb*9 genes have been deposited in GenBank under accession numbers AY518319 and AY518320, respectively

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development of floral papillae [9], a type of conical cells and multicellular trichomes [10]. Cotton fibers are single-cell trichomes that differentiate from individual epidermal cells of a developing seed (ovule). It is possible that MYB proteins may also play a role in regulating the differentiation and development of cotton fibers. Recently, six *Myb* cDNAs (*GhMyb*1–6) have been isolated from a cotton (*Gossypium hirsutum* L. cv. Acala SJ-2) ovule cDNA library [11]. All these six *Myb* genes are expressed in fiber cells; however, the expression patterns of these genes are different and can be grouped into two types. The type I including *GhMyb*1, 2, and 3 are abundantly expressed in all tissues, whereas the type II containing *GhMyb*4, 5, and 6 have comparatively lower expression levels, but exhibit a tissue-specific expression pattern. Suo et al. [12] have recently isolated another cotton R2R3-Myb gene, *GhMYB*109, which has a fiber-specific expression pattern. Both Northern analysis and in situ RNA hybridization indicate that *GhMYB*109 is specifically expressed in fiber initials and elongated fiber cells. Based on the expression patterns of these *Myb* genes in fiber cells, they might play an important role in the differentiation and development of cotton fibers.

We have previously isolated and characterized three cotton lipid transfer protein genes, *Ltp*3, 6, and 12, in our laboratory [13–15]. The temporal expression pattern of *Ltp*3 and *Ltp*6 in fiber cells suggests that they may play an important role in fiber development during the fiber elongation step. Systematic promoter analysis indicates that the 5′-flanking regions of *Ltp*3 (nt −614 to −300, relative to the translation initiation site) [16] and *Ltp*6 (nt −447 to −274) [17] can direct the expression of the *GUS* gene in leaf trichomes of transgenic tobacco plants. These results suggest that the 5′-flanking region of *Ltp*3 and *Ltp*6 might contain the fiber-specific promoter and regulatory elements.

In this study, we report the isolation and characterization of two fiber *Myb* cDNAs (*GhMyb*7 and 9) and their corresponding genes from cotton (*G. hirsutum* L. cv. DES119). The *Escherichia coli*-expressed GhMYB7 protein is able to bind to the promoter region (nt −614 to −580) of the *Ltp*3 gene. We also report the influence of GhMYB7 on plant and trichome development using transgenic tobacco and *Arabidopsis* plants which overexpress the *GhMyb*7 gene.

2. Materials and methods

2.1. Plant materials

Cotton (*G. hirsutum* L. cv. DES119) plants were grown in a greenhouse at the Agricultural Research Service (ARS) of USDA or annually planted in the field at the North Farm at Mississippi State University. Cotton flowers were tagged on the day of anthesis (0 DPA), and fibers were collected at different developmental stages (5, 10, 15, and 20 DPA).

2.2. Isolation of cotton R2R3-Myb genes

Total RNAs were isolated from 15 DPA fibers using a modified method of Hughes and Galau [18] and used for the construction of an adaptor-ligated cDNA library with a Marathon cDNA Amplification Kit (BD Biosciences, Clontech). The fiber cDNA library was used as a template for PCR amplification with two primers, a degenerate forward primer (5′-AAGGAATTCAGTTGYMGATTAAGATGG-3′) and a reverse primer (5′-GGAAGCTTRTCTGTTCKKCCTGG-3′), designed from the conserved region of R2 and R3 repeats of plant R2R3-MYB family, respectively. PCR was carried out for 35 cycles, with each cycle consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min. The 190-bp PCR product was excised from the gel, purified using a gel extraction kit (Qiagen), and cloned into a pGEM-T easy vector (Promega). The recombinant pGEM-T easy plasmids were sequenced using an ABI PRISM 310 DNA Genetic Analyzer. Based on the DNA sequence of the 190-bp PCR fragment, two full-length cDNAs, designated as *GhMyb*7 and *GhMyb*9, were cloned using the rapid amplification of cDNA ends (RACEs) method with the Marathon cDNA Amplification Kit (Clontech). The first 5′-RACE was conducted by PCR amplification of adaptor-ligated fiber cDNAs using a gene-specific primer (Myb-4) (5′-TAGATGTTTGGCTATCTGTGCCCATCTG-3′) and the adaptor primer AP1 (5′-CCATCCTAATACGACTCACTA-TAGGGC-3′). The amplification was carried out with initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 68 °C for 4 min, and final extension at 68 °C for 5 min. The first PCR product was then used as a template for the second nested PCR, which was carried out using the Myb-4 primer and another adaptor primer AP2 (5′-ACTCACTA-TAGGGCTCGAGCGGC-3′) under same conditions as the first PCR. The 3′-RACEs were performed as 5′-RACEs, except using a different gene-specific primer (Myb-3) (5′-ATAAATTACCTCAGACCTGATTTGAAACG-3′). The PCR products were purified, cloned, and sequenced as previously described. The 5′- and 3′-flanking regions of *GhMyb*7 and 9 genes were cloned using a PCR-based genomic walking method [19]. Cotton genomic DNA (3 μg) was individually digested with *Sca*I or *Xba*I, blunt-ended with T4 DNA polymerase, and ligated with the Marathon adaptor (Clontech). The adaptor-ligated genomic DNAs were used as templates for PCR amplifications by the same procedures as 5′- and 3′-RACEs. For 5′-genomic DNA walking, Myb-8 (5′-GCAAGCTTAGGGTCCCAGCTGCTTGAAGGA-3′) and AP1 primers were used for the first PCR amplification and Myb-4 and AP2 primers for the second (nested) PCR. For 3′-genomic walking, Myb-7 (5′-ATGGATCCGATGGGGCAC-CATTCTTGTTGCAAC-3′) and AP1 primers were for the first PCR, and Myb-3 and AP2 primers for the nested PCR. The nested PCR products were purified, cloned, and sequenced as previously described. The full-length *GhMyb*7 and *GhMyb*9 cDNAs and their corresponding genes were

amplified with *Pfu* DNA polymerase (Stratagene) and cloned into pGEM-T easy vector (Promega). At least two individual clones from each recombinant construct were sequenced to further confirm sequence accuracy of *GhMyb*7 and *GhMyb*9 genes.

2.3. Northern and genomic Southern analysis

For Northern analysis, total RNAs (10 μg) isolated from different cotton tissues, including leaves, flowers, roots, and fibers at different developmental stages (5, 10, 15, and 20 DPA) were electrophoresed on a formaldehyde agarose gel, transferred on a nylon membrane (GeneScreen Plus, Du Pont, Inc.), and hybridized with the C-terminal transregulatory region (TRR) of *GhMyb*7 cDNA labeled by [α-^{32}P] dCTP with the random priming method [20]. Hybridization and signal detection were carried out according to the manufacturer's instructions. Hybridization was performed in a buffer containing 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.4), 50% (v/v) formamide, 1% SDS, 5× Denhardt's solution (0.1% PVP-40, 0.1% BSA, and 0.1% Ficoll-400), 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. After hybridization, the membrane was washed three times with 2× SSPE for 10 min at room temperature, followed by two washes with 2× SSPE, 2% SDS at 65 °C for 15 min and two more washes with 0.1× SSPE at room temperature. The hybridization signal on the membrane was then detected by autoradiography. Genomic DNA for Southern analysis was prepared from cotton leaves by a modified method of Paterson et al. [21]. Ten micrograms of genomic DNA was individually digested with six restriction enzymes, *Dra*I, *Eco*RI, *Eco*RV, *Sca*I, *Ssp*I, or *Xba*I, and then electrophoresed on an agarose gel. The membrane transferring and hybridization were carried out under same conditions as in Northern analysis.

2.4. Expression and purification of fusion protein

The full-length and C-terminal truncated *GhMyb*7 cDNAs were cloned into pET-32b(+) (Novagen) at *Bam*HI and *Hin*dIII sites. The recombinant pET plasmids were transferred into *E. coli* AD494 (DE3) cells, and His-fusion proteins were induced by 1 mM IPTG. The fusion proteins were purified with a nickel-nitrilotriacetic acid (Ni-NTA) affinity column under denatured condition and then analyzed by SDS-PAGE. The fusion protein was also induced from the pET-32b(+) vector without the inserted gene, purified, and served as a negative control. The concentration of His-fusion proteins was determined by the Bradford method (Bio-Rad).

2.5. Gel mobility shift assay

The gel mobility shift assay (EMSA) was performed as described by Revizin [22]. The DNA–protein binding reaction was carried out by incubating 4 ng of ^{32}P-labeled *Ltp*3 promoter fragment (71 bp, nt −614 to −544) with 5 μg of GhMYB7 fusion protein and 0.5 μg of polydI·dC (Sigma). The binding reaction was then analyzed by electrophoresis on a 6% (w/v) nondenaturing polyacrylamide gel at 4 °C in a running buffer containing 0.5× TBE. The gel was dried onto a sheet of 3 MM paper (Whatman), and the retarded band (DNA–protein complex) was then detected by autoradiography.

The GhMYB7 binding site in the 71-bp *Ltp*3 promoter region was determined by further dissecting the promoter region. Two sets of PCR primers, Ltp3-F1 (5′-TTTAAATCC-TATTGTAGTGT-3′)/Ltp3-R1 (5′-TTTTTTTTATAAATAA-CACT-3′) and Ltp3-F2 (5′-TGAGAAAAGATAAAAATA-3′)/Ltp3-R2 (5′-CAAATATTAATATAAAGGTATTT-3′), were designed and used to amplify two DNA fragments, F1 (35 bp) and F2 (36 bp), corresponding to the *Ltp*3 promoter region from nt −614 to −544 (see Fig. 6A). The F1 and F2 fragments were electrophoresed, purified, and end-labeled with [γ-^{32}P] ATP (4500 Ci/mmol, ICN Biomedicals) by T4 polynucleotide kinase (NEB). The radiolabeled promoter fragments were further purified with MicroSpin G-50 columns (Amersham Pharmacia Biotech) and then used as DNA probes in EMSA.

2.6. Genomic origin analysis

The genomic origin of *GhMyb*7/*GhMyb*9 was determined by PCR amplifications of genomic DNAs isolated from diploid and allotetraploid *Gossypium* species. *G. herbaceum* (diploid A_1 genome, accession number A_1-57), *G. arboreum* (diploid A_2 genome, accession number A_2-86), *G. thurberi* (diploid D_1 genome, accession number D_1-1), *G. raimondii* (diploid D_5 genome, accession number D_5-4), and *G. hirsutum* L. cv. DES119 (allotetraploid AADD genome) were used as templates for PCR amplifications. PCR amplifications were carried out for 30 cycles using 0.5 μg genomic DNA of a selected *Gossypium* species and GhMyb7-PA1 (5′-AGCAAGCTTAGGTAGCTAGCATGCA-3′) and GhMyb7-PA5 (5′-CCCGTCGACTTTTTTTTTCTTTTCT-CTC-3′) primers, with each cycle consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The PCR products were analyzed on a 1% (w/v) agarose gel.

2.7. Effect of auxin on GhMyb7 expression

The effect of auxin on *GhMyb*7 expression was determined with an in vitro cotton ovule culture system [23]. Cotton ovules (0 DPA) were collected and cultured as described by Beasley [23]. For auxin treatment assay, the ovules were cultured in media containing 2 μM gibberellin (GA_3) and different concentrations (0, 1, 5, 10, 15, or 20 μM) of auxin (indole-3-acetic acid, IAA) for 10 days. For the time course study, the ovules were cultured in a basal

medium containing 2 μM GA_3 and 2 μM IAA. After incubation for 10 days, extra IAA was added into the culture at a final concentration of 10 μM, and ovules were then cultured for additional 1, 2, and 3 days. Total RNAs were isolated from fiber cells on the ovules cultured under different auxin concentrations or at different time periods, and then used as sources for the determination of the *GhMyb*7 transcript level by RT-PCR. The first-strand cDNAs were synthesized using SUPERSCRIPT II reverse transcriptase (Life Technologies) and then used as templates for PCR amplifications using Myb-3 and Myb-8 primers. The PCR amplifications were carried out with an initial denaturation at 95 °C for 2 min, 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 5 min. The RT-PCR products were electrophoresed on a 1% agarose gel, transferred into a Nylon membrane, and analyzed by Southern blotting using the ECL direct nucleic acid labeling and detection system (Amersham). For the internal control, a PCR reaction was also performed using a plant 18S rRNA primer pair (5 μM, Ambion) which replaced the *GhMyb*7-specific primers.

2.8. Overexpression of GhMyb7 in transgenic plants

The *GhMyb*7 coding region was amplified by PCR with GhMyb7-E1 (5′-GCTCTAGAATGGGGCACCATTCTTGTTG-3′) and GhMyb7-TH2 (5′-ATGGATCCTAAGGGTCCCAGCTGCTTG-3′) primers and cloned into a binary vector pBI121 (Clontech) downstream of CaMV 35S promoter to replace the GUS reporter gene. The recombinant plasmids were then introduced into *Agrobacterium tumefaciens* LBA4404 cells using a freeze–thaw method [24]. The recombinant *A. tumefaciens* LBA4404 cells were used to transform tobacco (*Nicotiana tabacum* L.) cells and *A. thaliana* (ecotype Landsberg *erecta*) using the standard *Agrobacterium*-mediated leaf-disk transformation method [25,26] and the floral dip transformation method [27], respectively.

3. Results

3.1. Cloning and characterization of GhMyb7 and GhMyb9 genes

Two full-length fiber cDNAs, *GhMyb*7 and *GhMyb*9 (Gh represents *G. hirsutum*), encoding plant R2R3-MYB proteins were isolated by PCR amplification using primers based on the conserved R2/R3 regions and the RACE method. Two genomic DNA fragments (2.77 and 2.67 kb) containing *GhMyb*7 and *GhMyb*9 genes were cloned using the genomic walking method. The nucleotide (nt) and deduced amino acid (aa) sequences of *GhMyb*7 and *GhMyb*9 genes along with their flanking regions are shown in Fig. 1. Both *GhMyb*7 and *GhMyb*9 encode a MYB protein containing 352 aa. These two GhMYB proteins share about 98.6% similarity at the aa level and exhibit general characteristics of plant MYBs: a conserved R2R3 DNA-binding domain (DBD) at the N-terminus with regularly spaced tryptophan (W) residues and a non-conserved transcriptional regulatory region (TRR) at the C-terminus of proteins. Like most plant MYB proteins, the DNA-binding domain (104 aa) of GhMYB7 and GhMYB9 proteins consists of two imperfect (R2 and R3) repeats (53 and 51 aa for each repeat, respectively). Both GhMYB7 and GhMYB9 contain an acidic TRR1 region (40 aa immediately downstream of the DNA-binding domain) located immediately downstream from their DNA-binding domains, and also have an overall acidic TRR region in the C-termini of proteins, suggesting that the region might act as a transcriptional activator. Both *GhMyb*7 and *GhMyb*9 contain two introns located at the N-terminal DBD regions, and these two genes share 88.3% identity in the nucleotide sequences. High level of identity in nucleotide sequences suggests that *GhMyb*7 and *GhMyb*9 genes are alloalleles in the allotetraploid cotton (*G. hirsutum* L. cv. DES119) genome (AADD genome) and derived from different genomic origins (see below). Interestingly, an auxin response element (AuxRE) (5′-GGTCCCAT-3′) (nt −386 to −379, relative to the translational ATG start site) and an auxin response factor 1 (ARF 1) binding site (5′-TGTCTC-3′) (nt −75 to −70) have been found in the 5′-flanking region of the *GhMyb*7 gene. In contrast, the *GhMyb*9 gene contains an AuxRE only (nt −346 to −339), and the ARF binding site is absent in its 5′-flanking region. Genomic Southern analysis of the allotetraploid cotton (*G. hirsutum* L. cv. DES119) genomic DNA digested with six restriction enzymes, *Dra*I, *Eco*RI, *Eco*RV, *Sca*I, *Ssp*I, and *Xba*I, showed that one to two DNA fragments hybridized to the *GhMyb*7-specific probe (Fig. 2), indicating that the *GhMyb*7 gene contained at least two copies in the cotton genome.

A phylogenetic tree (Fig. 3) constructed by comparing the derived aa sequences of GhMYB7 and GhMYB9 with other plant R2R3-MYBs reveals that GhMYB7 and GhMYB9 are grouped with *Arabidopsis* AtMYB26. AtMYB26 cannot be classified into the 22 subgroups of the *Arabidopsis* R2R3-MYB family [3,4] due to the lack of conserved motifs in the C-terminus of AtMYB26. GhMYB7 and GhMYB9 share about 96.5 and 97.4% aa identities, respectively, in the R2R3 conserved regions with AtMYB26, but only 40.9 and 41.2% aa identities, respectively, in the full-length coding region with AtMYB26. High levels of identity in the DBD regions among GhMYB7, GhMYB9, and AtMYB26 suggest that these three proteins might recognize a similar DNA-binding sequence.

3.2. Genomic origin analysis of GhMyb7 and GhMyb9 genes

Cotton (*G. hirsutum* L.), the predominant cultivated cottons, is an allotetraploid species of the AD genome and diverse from an ancient cross between Old World (diploid species of the A genome) and New World (diploid species of the D genome) cottons [28]. The genomic origins of

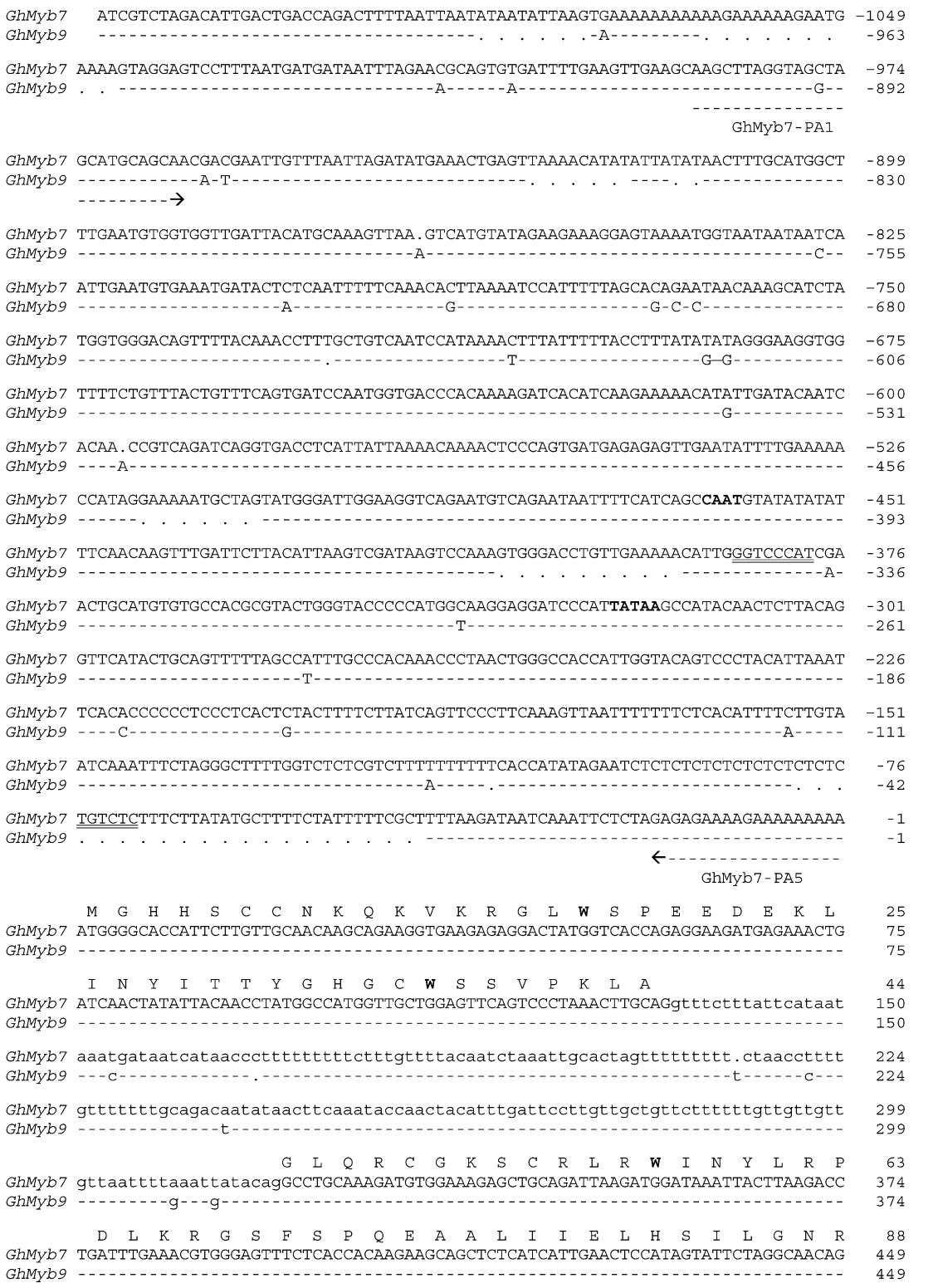

Fig. 1. Comparison of nucleotide and derived amino acid sequences of cotton *GhMyb*7 and *GhMyb*9 genes. The basal promoter elements (TATA and CAAT boxes) and the polyadenylation signal (AAATATA) are bolded. The putative auxin-response element (AuxRE) (GGTCCCAT) and the binding site of auxin response factor 1 (ARF1) (TGTCTC) are double-underlined. The sequences of two introns are presented in lowercase letters. The translational stop codon is marked with an asterisk. The first nt (A) of the translation start codon (ATG) is assigned as position 1 in the nt sequence, and the nt positions upstream of position 1 are presented with minus numbers. The identical nucleotides are represented with dash lines, and the gaps are marked as dot lines after aligning two genes. The conserved R2 and R3 imperfect repeat regions are located at aa 12–64 and aa 65–115, and the regularly spaced tryptophan (W) residues of each repeat are bolded. Three codons encoding different amino acids between coding regions of GhMYB7 and GhMYB9 proteins are underlined. Two PCR primers, GhMyb7-PA1 and GhMyb7-PA5 used in the determination of genomic origins are labeled.

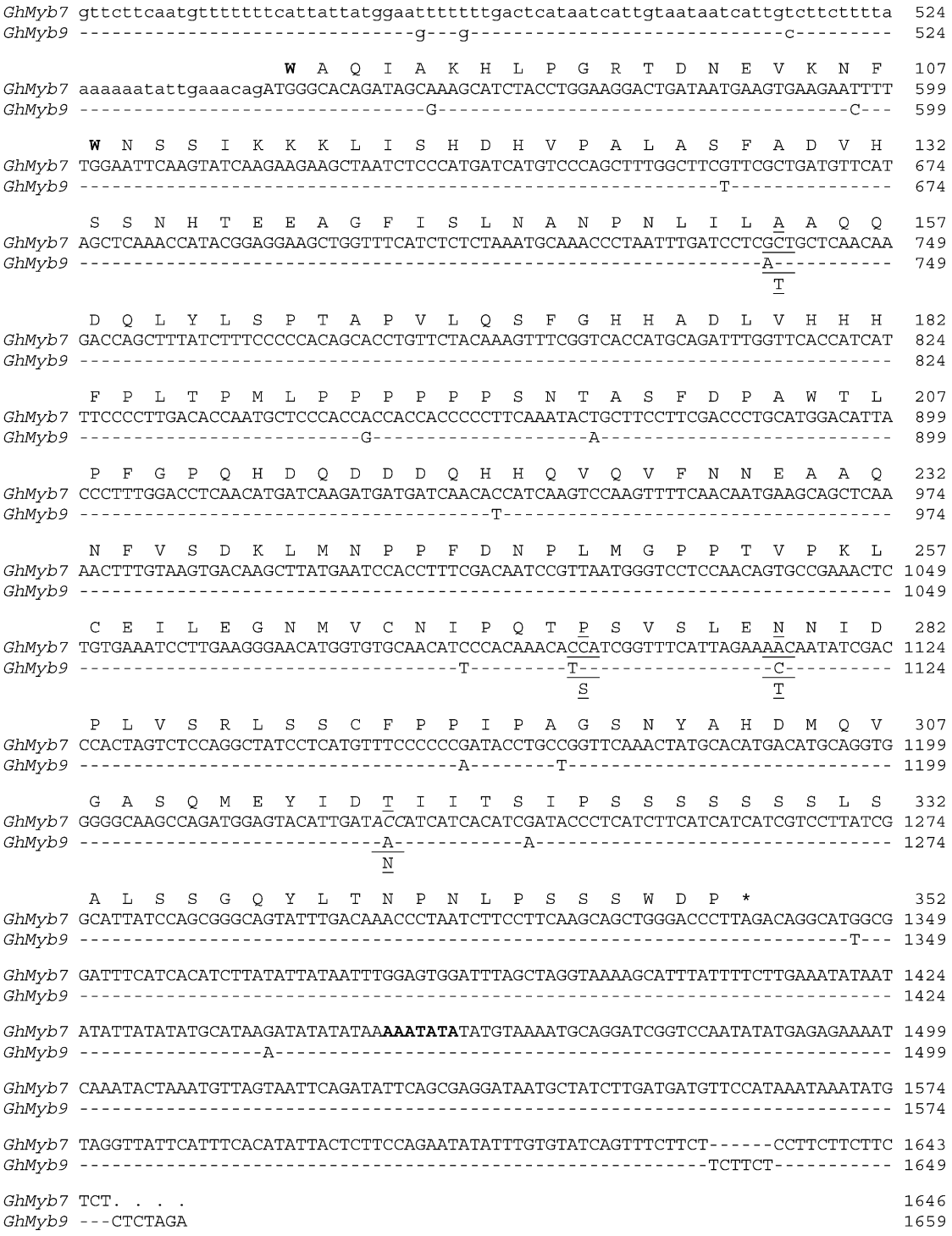

Fig. 1. (*Continued*).

*GhMyb*7 and *GhMyb*9 genes (Fig. 4) were analyzed by PCR amplifications of genomic DNAs isolated from *G. herbaceum* (diploid A_1 genome, accession number A_1-57), *G. arboreum* (diploid A_2 genome, accession number A_2-86), *G. thurberi* (diploid D_1 genome, accession number D_1-1), *G. raimondii* (diploid D_5 genome, accession number D_5-4), and *G. hirsutum* L. cv. DES119 (allotetraploid AADD genome) using GhMyb7-PA1 and GhMyb7-PA5 primers (Fig. 1). A 991-bp DNA fragment of the *GhMyb*7 gene was amplified from the genomic DNAs of *G. arboreum* (diploid A_2 genome) and *G. hirsutum* L. cv. DES119 (allotetraploid AADD genome), whereas a 909-bp *GhMyb*9 fragment was amplified from *G. raimondii* (diploid D_5 genome) and *G. hirsutum* L. cv. DES119 (allotetraploid AADD genome). A

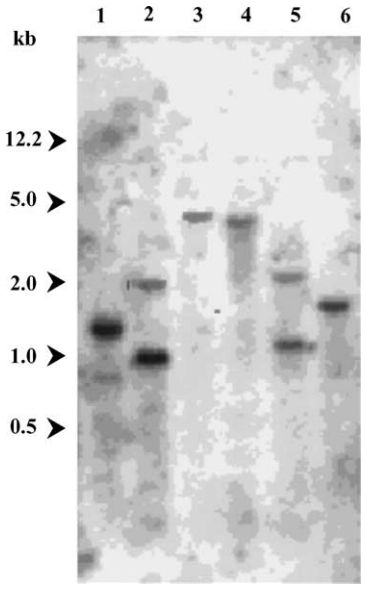

Fig. 2. Genomic Southern analysis of *GhMyb*7/9. Cotton genomic DNA (10 μg) was digested with *Dra*I (lane 1), *Eco*RI (lane 2), *Eco*RV (lane 3), *Sca*I (lane 4), *Ssp*I (lane 5), and *Xba*I (lane 6) and then hybridized with the TRR region of *GhMyb*7.

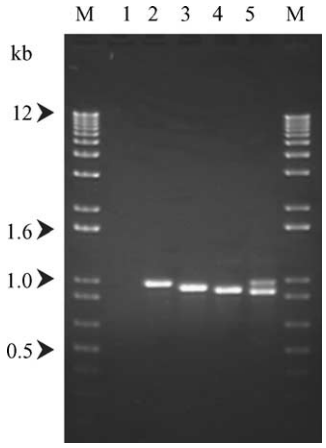

Fig. 4. PCR analysis of genomic origins of the *GhMyb*7 and *GhMyb*9 genes. Two gene-specific primers (GhMyb7-PA1 and GhMyb7-PA5) corresponding to *GhMyb*7/*GhMyb*9 were used in PCR amplifications of genomic DNAs isolated from five different cotton species: *G. herbaceum* (diploid A_1 genome) (lane 1), *G. arboreum* (diploid A_2 genome) (lane 2), *G. thurberi* (diploid D_1 genome) (lane 3), *G. raimondii* (diploid D_5 genome) (lane 4), and *G. hirsutum* L. cv. DES119 (allotetraploid AADD genome) (lane 5). Lane M represents 1 kb plus DNA ladder.

950-bp DNA fragment was also amplified from *G. thurberi* (diploid D_1 genome), indicating that *G. thurberi* (diploid D_1 genome) contains a homologous gene of *GhMyb*7 and *GhMyb*9. These results indicate that *GhMyb*7 and *GhMyb*9 are alloallelic genes in the allotetraploid cotton genome (AADD), but derived from different genomic origins, A_2 and D_5 subgenomes, respectively.

3.3. Temporal expression of GhMyb7/9 in fibers from different developmental stages

Northern blot analysis was used to examine the expression pattern of *GhMyb*7/9 gene in fibers and other cotton tissues. The non-conserved TRR region of *GhMyb*7/9 was used as a hybridization probe in order to prevent cross-hybridization with other cotton *Myb* genes in the Northern analysis. Northern results (Fig. 5) showed that the *GhMyb*7/9 probe hybridized to a 1.35-kb transcript, which was only

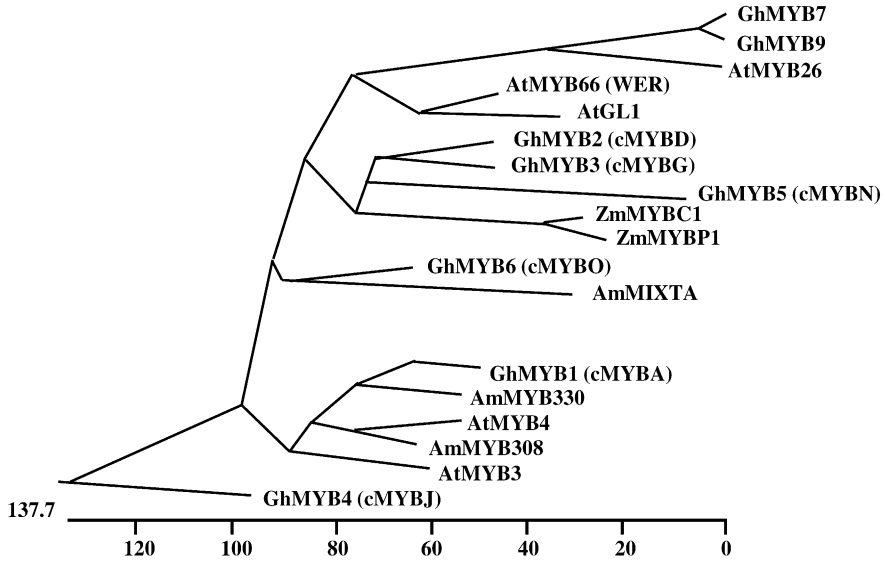

Fig. 3. A phylogenetic tree constructed by comparing amino acid sequences of R2R3-MYB proteins from four different plant species. The phylogenetic tree was constructed by the Clustal method using the MegAlign program (DNASTAR, Inc.) based on the amino acid sequences of GhMYB7, 9, and 16 other plant R2R3-MYB proteins, including AtGL1 [8], AtMYB66 (WER) [56], AtMYB3 [3], AtMYB4 [3], and AtMYB26 [3] from *A. thaliana*, ZmMYBC1 [57] and ZmMYBP1 [58] from maize (*Zea mays*), AmMYB308 and AmMYB330 [45] from snapdragon (*A. majus*), and GhMYB1, 2, 3, 4, 5, and 6 from *G. hirsutum* [11].

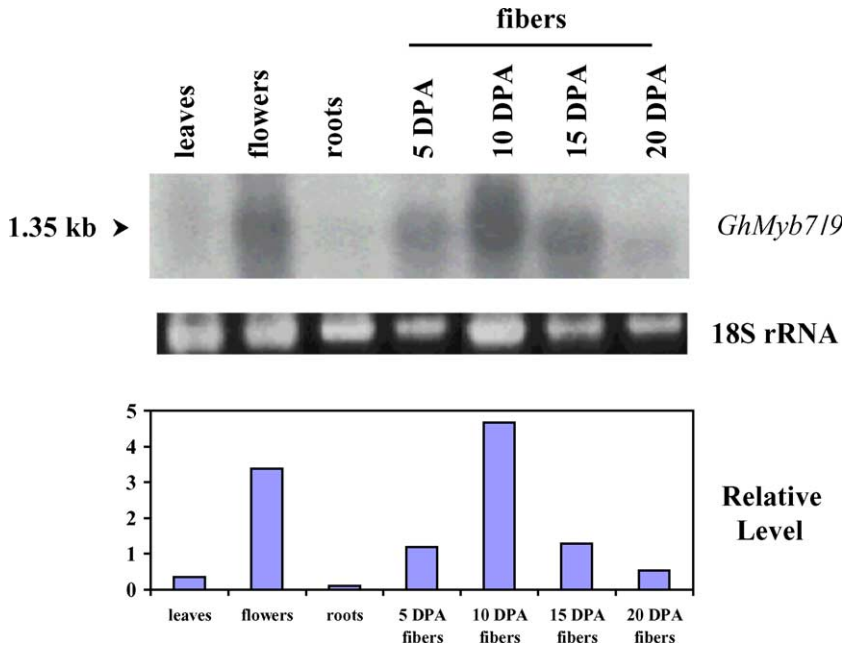

Fig. 5. Northern blot analysis of *GhMyb*7/9. Total RNA (10 μg) isolated from leaves, flowers, roots, and fibers at 5 DPA, 10 DPA, 15 DPA, and 20 DPA were electrophoresed on an agarose gel and hybridized with the TRR region of *GhMyb*7. The EtBr-stained RNA gel was included as a control of loading. The relative transcript levels of *GhMyb*7/9 were determined by the ratio of hybridized intensity of the 1.35-kb *GhMyb*7 transcript to the EtBr-stained 18S rRNA band using Scion Image program (Scion Corporation, http://www.sciocorp.com).

detected in flowers and fibers and was absent in both leaves and roots. The *GhMyb*7 expression in fiber cells showed a developmentally regulated manner; its expression level reached a maximum at 10 DPA (the elongation stage) and gradually decreased from 15 to 20 DPA (the secondary cell wall synthesis stage). The temporal expression pattern of *GhMyb*7/9 gene in developing fiber cells suggests that *GhMyb*7/9 might play a role in the regulation of gene expression during fiber development.

3.4. Interaction between Ltp3 promoter and GhMYB7 protein

A 71-bp Ltp3 promoter (nt −614 to −544, relative to translation initiation site, ATG, Fig. 6A) was radiolabeled and used as a probe to determine its interaction with the GhMYB7 protein by gel mobility shift assay (EMSA). The EMSA results (Fig. 6B) showed that DNA–protein complexes (retarded bands) were formed in the binding reactions containing the full-length GhMYB7 protein (GhMYB7-1056) and C-terminal truncated GhMYB7 protein (GhMYB7-717). No complex was present in the reactions containing free probe only and the Trx·Tag protein from pET-32b(+) vector. The EMSA results suggest that the *Ltp*3 promoter region (nt −614 to −544) contains a DNA motif specifically recognized by the GhMYB7 protein, and the N-terminal region (including DNA-binding domain) of GhMYB7 is sufficient for specific DNA binding. The results also indicate that the DNA-binding ability of the C-terminal truncated GhMYB7 protein (containing the DNA-binding domain only) is much stronger than that of the full-length GhMYB7 protein.

A putative GhMYB7 binding site, GTTA, was located at nt −596 to −593 within the 71-bp *Ltp*3 promoter region (nt −614 to −544) (Fig. 6A). This 71-bp promoter region (*Ltp*3-70) was further divided into two small fragments, *Ltp*3-70-F1 (nt −614 to −580) and *Ltp*3-70-F2 (nt −579 to −544), which were used in the binding assay with GhMYB7. The EMSA assays (Fig. 6C and D) showed that the DNA–protein complex (retarded band) was only present in the reaction containing the *Ltp*3-70-F1 fragment as the probe, but absent with the *Ltp*3-70-F2 probe. These data suggest that the GhMYB7 protein may recognize and bind the DNA motif, ''5′-GTTA-3′'', in the *Ltp3* promoter.

3.5. Expression of GhMyb7/9 in fiber cells was induced by auxin

An in vitro ovule culture system [23] was used to study the effect of auxin on the expression of the *GhMyb*7 gene to eliminate the level of endogenous auxin. Fiber initials were formed when 0 DPA ovules were grown in a GA_3-containing medium for 1–2 days; however, the ovules eventually produced calli instead of lint fibers after 10-day incubation. In the presence of GA_3 and IAA, the ovules were able to produce lint fibers, and fiber production increased with higher concentration of IAA. The fiber production reached a maximum with 10 μM IAA and 2 μM GA_3; however, fiber production decreased when the IAA concentration was higher than 10 μM. The expression of the *GhMyb*7/9 gene

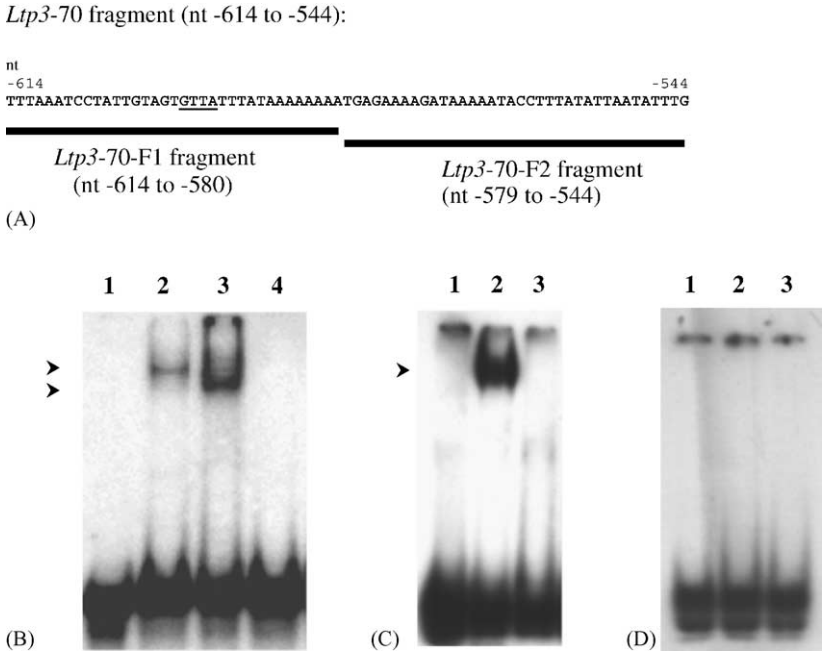

Fig. 6. Electrophoretic mobility shift assays (EMSA) of interactions between the *Ltp*3 promoter fragments and GhMYB7 protein. The corresponding positions of two promoter elements (*Ltp*3-70-F1 and *Ltp*3-70-F2) in the *Ltp*3-70 promoter region are indicated and the GTTA sequence in *Ltp*3-70-F1 is underlined (panel A). The ^{32}P-labeled *Ltp*3-70 (nt −614 to −544) (panel B), *Ltp*3-70-F1 (nt −614 to −580) (panel C), and *Ltp*3-70-F2 (nt −579 to −544) (panel D) fragments were used in the binding assay with the GhMYB7 protein or the C-terminal truncated GhMYB7-717 protein. Lanes 1, 2, 3, and 4 in panel B contain free probe only, GhMYB7, GhMYB7-717 (C-terminal truncated), and Trx·Tag protein (pET-32b(+) control), respectively. Lanes 1, 2, 3 in panels (C) and (D) contain free probe, GhMYB7, and Trx·Tag protein, respectively. Arrowhead indicates the DNA–protein complex.

was also induced in the in vitro cultured fibers (Fig. 7A). The *GhMyb*7/9 transcript was detected when the IAA concentration was higher than 5 μM, and the transcript level reached a maximum in the presence of 10 μM IAA. However, *GhMyb*7/9 expression decreased when the IAA concentration was higher than 10 μM. Because lint fiber production is increased with the induced expression of *GhMyb*7/9 transcript, *GhMyb*7/9 might play a role in promoting the synthesis of lint fibers in the ovule culture system.

In the time course assay, *GhMyb*7/9 expression increased after 1- to 2-day incubation with the addition of 10 μM IAA to the ovule culture; however, *GhMyb*7 expression slightly decreased after 3-day incubation (Fig. 7B). Similar to the field-grown cotton, *GhMyb*7/9 expression in fiber cells grown in vitro was also developmentally regulated; the *GhMyb*7/9 transcript level gradually increased from 11 DPA to 13 DPA (Fig. 7B). Since fiber development is temperature-dependent [29], the development therefore might be different for fiber cells grown in vitro and in the field. The maximal *GhMyb*7/9 transcript level was found in 10-DPA fiber cells from field-grown cotton. In contrast, the *GhMyb*7/9 transcript in fiber cells from in vitro ovule culture still increased after 10 DPA. The *GhMyb*7/9 expression, however, was regulated in a temporal pattern under both growth conditions.

Combining the expression data of *GhMyb*7/9 under different concentrations of IAA and from the time course study indicated that the expression of *GhMyb*7/9 was induced by the IAA hormone. Since the induction of *GhMyb*7/9 expression was observed after 1- to 2-day IAA treatments, *GhMyb*7/9 thus could not be classified as auxin early-responsive genes. Our data suggest that *GhMyb*7/9 expression and lint fiber production may require an appropriate concentration of IAA. A fine adjustment of auxin concentration in fiber cells may play an essential role in regulating *GhMyb*7/9 and consequently controlling fiber development. The decreased expression of *GhMyb*7/9 under higher IAA concentrations (more than 10 μM) and longer incubation time (after 3-day incubation) also suggest the possibility of feedback regulation between *GhMyb*7/9 expression and auxin.

3.6. Overexpression of GhMyb7 gene caused a pleiotropic effect on the development of transgenic plants

Agrobacterium-mediated transformation method was used to generate the transgenic tobacco and *Arabidopsis* plants carrying CaMV 35S:*GhMyb*7 transgene (overexpressing *GhMyb*7 gene). In comparison with the wild-type plants, both CaMV 35S:*GhMyb*7 transgenic tobacco and *Arabidopsis* plants exhibited the pleiotropic effect on the developmental process. The transgenic tobacco plants carrying CaMV 35S:*GhMyb*7 transgene were marked with some altered phenotypes, including dwarf, thinner stem, long-shape leaves, longer petioles, and fewer root branch

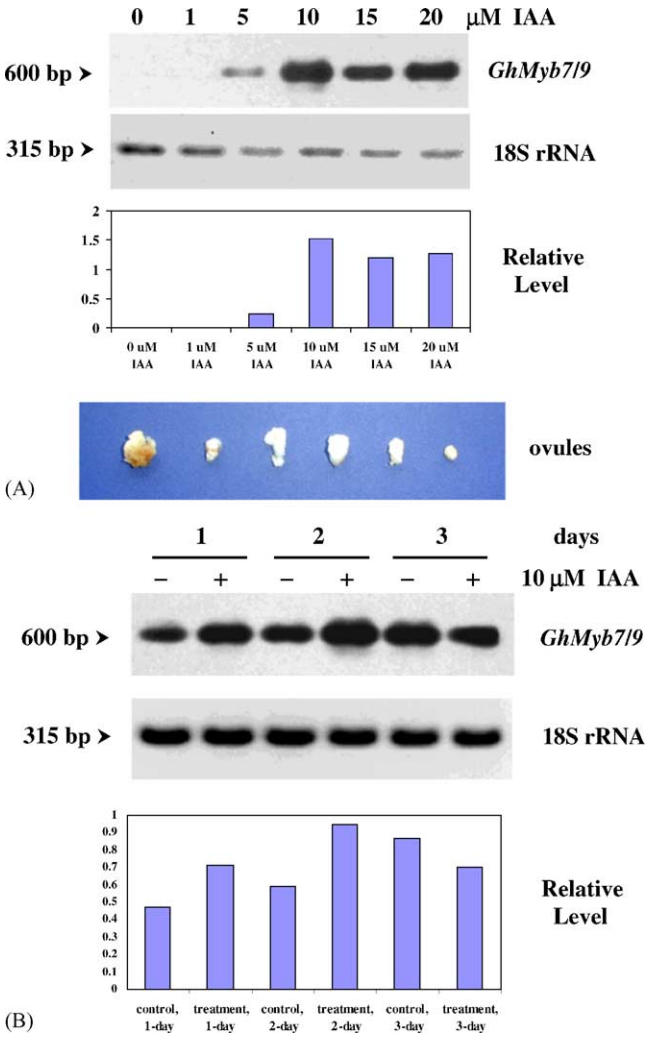

Fig. 7. Effect of IAA on *GhMyb*7 expression in fiber cells from in vitro ovule culture. Panel A shows *GhMyb*7 expression in response to different concentrations (0, 1, 5, 10, 15, and 20 μM) of IAA. Panel B shows the effect of culture time on *GhMyb*7 expression after adding 10 μM IAA to the 10 DPA ovule culture. Fiber RNA samples were isolated from ovules cultured in a medium containing 2 μM GA_3 along with different concentration of IAA (panel A) or isolated from 11–13 DPA ovules in the time course assay (panel B). Fiber RNAs were used as templates in RT-PCR amplifications with two *GhMyb*7-specific primers or a specific primer pair of plant 18S rRNA, respectively. The phenotypes of in vitro cultured ovules under different conditions are included. The relative transcript levels of *GhMyb*7/9 were determined by the ratio of hybridized intensity of the 600-bp TRR region of *GhMyb*7 transcript to the 315-bp 18S rRNA band using Scion Image program (Scion Corporation, http://www.sciocorp.com).

development (Fig. 8A). In addition to the phenotypes of dwarf and irregular leaf shapes, the CaMV 35S:*GhMyb*7 transgenic line also showed less apical dominance and delayed inflorescence formation after transplanting into soil pots (Fig. 8A). A single primary shoot apex was normally found in the wild-type plant; in contrast, the 35S:*GhMyb*7 transgenic line had at least two secondary shoot apexes along with the primary shoot apex. Under the long-day condition (LD, 16-h light and 8-h dark) at 22 °C, the wild-type plant started bolting and flowering after transplanting into a soil pot for one and half months; whereas the transgenic line under the same growth condition showed no sign of bolting. The flowering process of the transgenic line was delayed for at least 2 months. These results indicate that the overexpression of the GhMYB7 (a transcription factor) might influence multiple pathways to cause such a pleiotropic effect on plant development.

Similarly, the 35S:*GhMyb*7 transgenic *Arabidopsis* plants also showed extensive effects on plant development, and the altered phenotypes included dwarf, curly shape in rosette leaves, and retarded root development (Fig. 8B). Additionally, a high percentage of fatality was also observed in transgenic lines that overexpress the *GhMyb*7 gene (data not shown). The defect of root development might be a major factor resulting in fatality. The flowering process in 35S:*GhMyb*7 transgenic *Arabidopsis* plants was also investigated under both LD and short day (SD, 8-h light and 16-h dark) conditions. Under LD condition, the floral development in transgenic lines was delayed about 4 days in comparison with the wild-type plants. Furthermore, the extremely short floral stems were observed in transgenic lines when compared with those in the wild-type plant (Fig. 8B). The wild-type plant bolted at the age of 8 weeks old under SD condition; however, no sign of inflorescence was observed in transgenic lines at the same age (Fig. 8B). The inflorescence was absent in transgenic lines even at the age of 20 weeks old (Fig. 8B). These results suggest that constitutive overexpression of the *GhMyb*7 gene might inhibit the GA biosynthesis or signaling pathway in *Arabidopsis* and cause an impaired floral development under SD condition.

4. Discussion

The *R2R3-MYB* gene family is one of very large and diverse super-gene families in the plant kingdom. The diverse R2R3-MYB super family is only observed in plants, and they govern many ‘‘plant-specific’’ processes [7,30]. Several members of *R2R3-MYB* genes have been shown to control cotton fiber development [11,12,31]. In this study, two cotton *R2R3-MYB* genes (*GhMyb*7 and *GhMyb*9) were isolated and characterized. Our genomic origin analysis (Fig. 4) indicated that *GhMyb*7 and *GhMyb*9 are alloallelic genes in the allotetraploid cotton. Several studies [32–34] have suggested that the diploids *G. herbaceum* (A_1) and *G. raimondii* (D_5) are the A and D genome donors of the tetraploid *G. hirsutum* (AD), respectively. However, our genomic origin analysis (Fig. 4) showed that *GhMyb*7 gene was present in A_2 (*G. arboreum*) and AD (*G. hirsutum*) genomes, whereas *GhMyb*9 is in D_5 (*G. raimondii*) and AD (*G. hirsutum*) genomes, suggesting that *G. raimondii* (D_5) and *G. arboreum* (A_2) (not the A_1 genome from *G. herbaceum*) are likely the ancestral D genome and A genome donors for the allotetraploid *G. hirsutum* (AD genome).

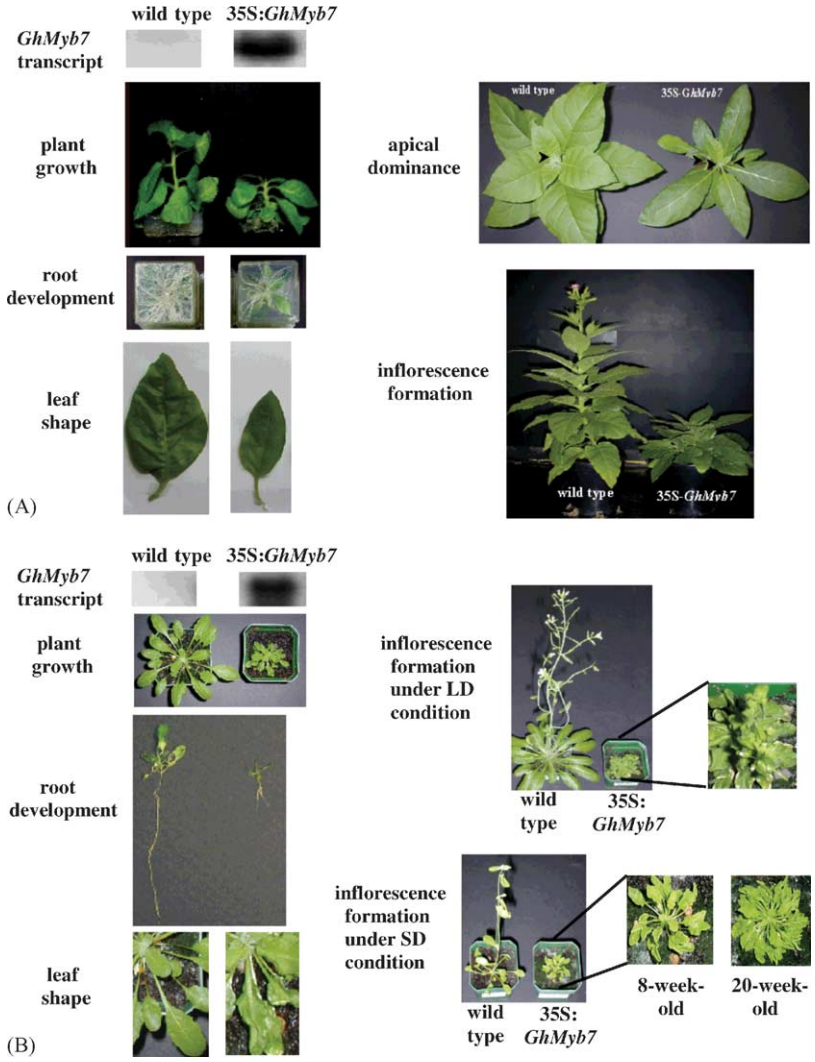

Fig. 8. Effect of *GhMyb*7 overexpression on the plant development and inflorescence formation of transgenic tobacco (A) and *Arabidopsis* (B) plants. The altered phenotypes, including dwarf, retarded root development, and the abnormal shape of leaves, were observed in transgenic plants carrying the CaMV 35S:*GhMyb*7 transgene. The altered phenotype of less apical dominance was also observed in the transgenic tobacco plant (A). The delayed inflorescence formation was shown in both transgenic tobacco (A) and *Arabidopsis* (B) plants under LD condition; however, the extremely short floral stems were found in the transgenic *Arabidopsis* plant. Under SD condition, the impaired floral development in the 8-week-old transgenic *Arabidopsis* plant was compared with the wild-type plant. The inflorescence was still absent in the 20-week-old transgenic line under SD condition. The wild-type tobacco and *Arabidopsis* plants at the same growth stage were included for comparison with transgenic plants that overexpress the *GhMyb*7 gene. The *GhMyb*7 transcript level in the wild-type plants and transgenic plants harboring CaMV 35S:*GhMyb*7 transgene was also included. Arrowheads indicate the shoot apexes.

Northern analysis (Fig. 5) showed that the expression of the *GhMyb*7/9 gene in fiber cells is developmentally regulated, and its transcript peaks at about 10 DPA (the early stage of fiber elongation). The Northern data, however, do not distinguish the expression between the two genes due to their sequence similarity. Cedroni et al. [35] have reported that two homologous *GhMYB*1 genes in allopolyploid cotton are differentially expressed. It will be interesting to examine whether this allelic-specific expression pattern also exists between *GhMyb*7 and *GhMyb*9. Sequence comparison between *GhMyb*7 and *GhMyb*9 indicates that there is less homology in their 5′-UTR regions. RT-PCR can be used to determine the individual expression of the two genes with gene-specific primers corresponding to their 5′-UTRs. Northern analysis indicates that *GhMyb*7/9 expression is also induced by exogenous auxin (Fig. 7A and B). Auxin plays an important role during the early stage of fiber elongation [36]. The IAA level in fiber cells was relatively low at 5 DPA and then increased at 10 DPA [37], which is consistent with the *GhMyb*7/9 expression pattern in developing fiber cells. The mechanism of auxin-induced gene expression has been recently proposed, which involves proteolysis via the ubiquitin-proteasome degradation pathway [38,39]. When the auxin level is low, Aux/IAA proteins dimerize with ARF (auxin-responsive factor) proteins. ARFs are transcriptional factors and activate the transcription of auxin-inducible genes by binding to TGTCTC-type AuxREs (auxin response elements) of auxin-inducible genes. The

formation of heterodimer of Aux/IAA and ARF will reduce the level of ARF and result in decreasing the transcription of auxin-inducible genes. The Aux/IAA proteins are degraded through the SCF^{TIR1}-associated ubiquitin-proteasome pathway promoted by high levels of auxin [38,39]. Several compositions of TGTCTC-type AuxREs have been found in different auxin-induced genes, including a sole "G/TGTCCCAT" element in the pea *PS-IAA*4/5 promoter [40], the "TGTCTC" element in the soybean *GH*3 promoter [41], and the dual "G/TGTCCCAT" and "TGTCTC" combination in the soybean *SAUR* promoter [42]. It has been suggested that the "G/TGTCCCAT" element might be a degenerate form of the "TGTCTC" element with the "*TGTCCAT*" sequence [43,44]. Here, the *GhMyb*7 and *GhMyb*9 genes contain the dual combination AuxRE and a TGTCTC-type AuxRE in their promoter regions, respectively, and the expressions of *GhMyb*7 and *GhMyb*9 were indeed induced by auxin as shown in Fig. 7A and B. It is well known that auxin mediates the processes of cell differentiation, division, elongation, and expansion in plants. Young developing tissues, such as leaves and roots, generally contain higher levels of auxin. Our preliminary data of promoter analysis showed that the 0.9-kb *GhMyb*7 promoter also directed GUS expression in the youngest leaves and meristem of transgenic tobacco plants (data not shown).

The EMSA assays showed that the *Ltp*3 promoter region from nt −614 to −580 interacts with the GhMYB7 protein (Fig. 6B and C). The EMSA results also showed that the N-terminal DNA-binding domain alone (GhMYB7-717) is sufficient to confer the DNA-binding ability. The truncated protein also exhibited a higher affinity with the *Ltp*3 promoter than the full-length GhMYB7 protein (Fig. 6B). It has been suggested that serine or threonine residues in the C-terminal domain of MYB proteins, which are possible substrates of kinases, might affect DNA-binding or transcriptional activation potential via phosphorylation [7,45]. The derived GhMYB7 amino acid sequence reveals that the C-terminus of the protein contains about 13% serine and 4.6% threonine, suggesting the possibility of higher DNA-binding affinity for the C-terminal truncated GhMYB7 protein (GhMYB7-717). The *Ltp*3 gene, encoding a lipid transfer protein, is specifically expressed in developing fiber cells and abundantly expressed during the fiber elongation stage [13,14]. The promoter region (from nt −614 to nt −299) of the *Ltp*3 gene has been shown to direct *GUS* expression specifically in trichome cells of transgenic tobacco plants [16]. In this study, we have tentatively identified a putative DNA-binding motif of GhMYB7, GTTA, located in the *Ltp*3 promoter region (nt −596 to −593) via EMSA assays. There are three types (I, II, and IIG) of DNA-binding domains for plant R2R3-MYB proteins [46]. Type I has the sequence T/CAACNG and is similar to c-MYB and animal MYB proteins [47]. Type II has the T/CAACT/AAC/AC sequence and type IIG has T/CACCT/AAC/AC. The majority of plant R2R3 MYB proteins preferentially bind to type II and type IIG. The putative GhMYB7 DNA-binding sequence GTTA contains the core motif (GG/TTa, or its complementary sequence tAA/CC) of the canonical binding site of R2R3-MYB proteins. Further experiments are needed to test whether GhMYB7 indeed binds to the GTTA sequence. The DNA element identified via in vitro binding, however, is only an indication of possible target site. An in vivo transient expression assay can be used to confirm the interaction between the GhMYB7 protein and *Ltp*3 promoter. The promoter region (nt −614 to −580) of *Ltp*3 is the first identified *cis*-element which might directly confer the fiber/trichome-specific activity. Based on the observations of auxin-induced expression of *GhMyb*7 and similar expression patterns of *GhMyb*7 and *Ltp*3 genes during fiber development, the GhMYB7 protein might play a role in the pathway of auxin-promoted fiber elongation by activating the expression of downstream genes.

The function of the *GhMyb*7 gene can be determined by its expression in transgenic plants using a constitutive 35S promoter or trichome (or fiber-specific) promoters. Constitutive overexpression of *GhMyb*7 in transgenic *Arabidopsis* and tobacco plants has resulted in no difference in the formation of leaf trichomes (data not shown). However, the overexpression has exhibited pleiotropic effects on plant development (Fig. 8A and B). The altered phenotypes in transgenic plants, including stunted growth, less apical dominance, small and narrow shaped tobacco leaves, curly shaped *Arabidopsis* rosette leaves, and slow root development have been observed. The overexpression of subgroup 4 *Myb* genes (*AtMyb*4, *AmMyb*308, *AmMyb*330, and *GhMyb*1) [3,4,48] have also shown similar phenotypes, such as stunted growth and the change of leaf shape in transgenic plants [49–51]. These studies suggest that the corresponding pleiotropic defects might be caused by the repression of lignin biosynthesis. However, the defects caused by *GhMyb*7 overexpression, such as less apical dominance and retarded root development, have not been observed in transgenic plants overexpressing those subgroup 4 *Myb* genes. The derived GhMYB7 protein sequence also does not have any structural similarity in the C-terminus with the subgroup 4 gene members. It is possible that *GhMyb*7 overexpression also affects some other pathways involved in plant development.

Interestingly, *GhMyb*7 overexpression also affects the flowering process in transgenic *Arabidopsis* plants (Fig. 8B). The flowering process in *Arabidopsis* has been proposed to be controlled by four factors: photoperiod, vernalization, autonomous response, and gibberellic acid (GA) [52]. These four factors interact with each other in response to different environmental conditions and developmental sequences to mediate the flowering process. However, it is believed that photoperiod response plays a major role in the control of flowering under the LD condition and GA is a predominant factor in mediating flowering under the SD condition in *Arabidopsis*. The altered phenotypes shown in 35S:*GhMyb*7 transgenic *Arabidopsis* plants, including dwarf stature, late-

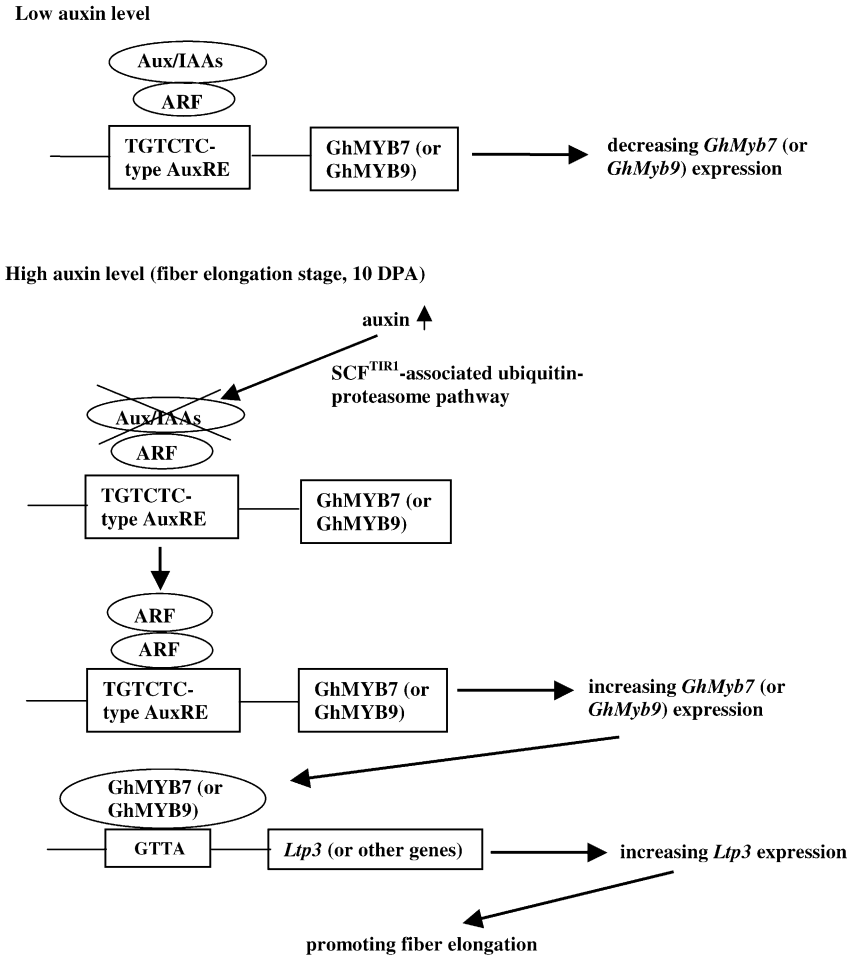

Fig. 9. Proposed role of GhMYB7 (or GhMYB9) in the regulation of *Ltp*3 gene expression during fiber elongation.

flowering under LD condition, and impaired floral development under SD condition, are very similar to those in several GA-defective mutant lines, such as *ga*1–3, *ga*4, and *ga*5 mutants [53]. These results strongly suggest that *GhMyb*7 overexpression might affect the GA biosynthesis or signaling pathway in *Arabidopsis* plants. The expression of the *GhMyb*7 gene itself is induced by auxin, and the gene product might mediate either the GA biosynthesis or signaling pathway. It will be interesting to investigate whether *GhMyb*7 plays a role in the mechanisms for cross-talk between two important hormones, auxin and GA, during plant growth and development.

The comparison of aa sequence of GhMYB7 with AtMYB26, an *Arabidopsis* R2R3-MYB protein, has showed that there is 96% homology in N-termini (DNA-binding domains) between the two proteins. *AtMyb*26 has not yet been classified into the 22 subgroups of the *Arabidopsis R2R3-MYB* gene family due to the lack of conserved motifs in the C-terminus of the AtMYB26 protein [3,4]. However, *AtMyb*26 gene has been recently reported to play a role in the lignin and cellulose deposition of endothecium cell walls of anther [54]. An *Arabidopsis* male sterile mutant caused by the disrupted *AtMyb*26 gene has a phenotype of non-dehiscent anthers due to the impairing secondary cell wall thickenings. The mutant line yet contains the functional pollens. Moreover, the change of three amino acids in the DNA-binding domain region of AtMYB26 is sufficient for the sterile phenotype. The high expression level of *GhMyb*7 transcript in cotton flowers (Fig. 5) and strong homology (96%) in the DNA-binding domain regions between GhMYB7 and AtMYB26 indicate that the *GhMyb*7 gene might also play a role in the inflorescence process. Recently, the *Arabidopsis* cDNA microarray has been successfully applied to reveal the activation of multiple regulatory pathways during cold acclimation [55]. The cDNA microarray technique might provide a better system to understand the affected pathways involved in plant development caused by *GhMyb*7 overexpression.

The possible role of *GhMyb*7 (or *GhMyb*9) in regulating the expression of the *Ltp*3 gene during fiber elongation stage is proposed (Fig. 9). In the early stage of fiber elongation, the expression of *GhMyb*7 (or *GhMyb*9) is downregulated by the dimerization between Aux/IAA and ARF proteins in the presence of low levels of auxin in fiber cells. The

dimerization reduces the amounts of ARF that is bound to the AuxRE element located upstream of the *GhMyb*7 (or *GhMyb*9) gene. In the later stage of fiber elongation (about 10 DPA), increased auxin levels promote the degradation of Aux/IAA proteins via the SCF^{TIR1}-associated ubiquitin-proteasome pathway. In the absence of Aux/IAAs, ARF can form a homodimer, which then binds to the AuxRE element to activate the expression of *GhMyb*7 (or *GhMyb*9). With increased expression, the GhMYB7 (or GhMYB9) protein may bind to a sequence-specific "GTTA" *cis*-element in the promoter region of the *Ltp*3 gene (or other genes) and transactivates the expression of *Ltp*3 (or other genes) to promote the process of fiber elongation. Further experiments are needed to determine the possibility of cooperation between GhMYB7 (or GhMYB9) and other transcription factors in regulating *Ltp*3 expression during fiber elongation.

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References

[1] T.J. Gonda, The c-Myb oncoprotein, J. Biochem. Cell Biol. 30 (1998) 547–551.
[2] K. Ogata, S. Morikawa, H. Nakamura, A. Sekikawa, T. Inoue, H. Kanai, A. Sarai, S. Ishii, Y. Nishimura, Solution structure of a specific DNA complex of the Myb DNA-binding domain with cooperative recognition helices, Cell 79 (1994) 639–648.
[3] H.D. Kranz, M. Denekamp, R. Greco, H. Jin, A. Levya, R.C. Meissner, K. Petroni, A. Urzaingui, M. Bevan, C. Martin, S. Smeekens, C. Tonelli, J. Paz-Ares, B. Weisshaar, Towards functional characterization of the members of the *R2R3-MYB* gene family from *Arabidopsis thaliana*, Plant J. 16 (1998) 263–276.
[4] R. Stracke, M. Werber, B. Weisshaar, The R2R3-MYB gene family in *Arabidopsis thaliana*, Curr. Opin. Plant Biol. 4 (2001) 447–456.
[5] P.D. Rabinowicz, E.L. Braun, A.D. Wolfe, B. Bowen, E. Grotewold, Maize R2R3 Myb genes: sequence analysis reveals amplification in the higher plants, Genetics 153 (1999) 427–444.
[6] J. Avila, C. Nieto, L. Canas, M.J. Benito, J. Paz-Ares, *Pentunia hybrida* genes related to the maize regulatory *C*1 gene and to animal *myb*-oncogenes, Plant J. 3 (1993) 553–562.
[7] C. Martin, J. Paz-Ares, MYB transcription factors in plants, Trends Genet. 13 (1997) 67–73.
[8] D.G. Oppenheimer, P.L. Hermn, S. Sivakumaran, J. Esch, D.M. Marks, A *myb* gene required for leaf trichome differentiation in *Arabidopsis* is expressed in stipules, Cell 67 (1991) 483–493.
[9] K. Noda, B.J. Glover, P. Linstead, C. Martin, Flower colour intensity depends on specialized cell shape controlled by a *Myb*-related transcription factor, Nature 369 (1994) 661–664.
[10] B.J. Glover, M. Perez-Rodriguez, C. Martin, Development of several epidermal cell types can be specified by the same MYB-related plant transcription factor, Development 125 (1998) 3497–3508.
[11] L.L. Loguercio, J.-Q. Zhang, T.A. Wilkins, Differential regulation of six novel *MYB*-domain genes defines two distinct expression patterns in allotetraploid cotton (*Gossypium hirsutum* L.), Mol. Gen. Genet. 261 (1999) 660–671.
[12] J. Suo, X. Liang, L. Pu, Y. Zhang, Y. Xue, Identification of *GhMYB109* encoding a R2R3 MYB transcription factor that expressed specifically in fiber initials and elongating fibers of cotton (*Gossypium hirsutum* L.), Biochim. Biophys. Acta 1630 (2003) 25–34.
[13] D.-P. Ma, H. Tan, Y. Si, R.G. Creech, J.N. Jenkins, Differential expression of a lipid transfer protein gene in cotton fiber, Biochim. Biophys. Acta 1257 (1995) 81–84.
[14] D.-P. Ma, H.-C. Liu, H. Tan, R.G. Creech, J.N. Jenkins, Cloning and characterization of a cotton lipid transfer protein gene specifically express in fiber cells, Biochim. Biophys. Acta 1344 (1997) 111–114.
[15] D.-P. Ma, H.-C. Liu, C.-Y. Hsu, Expression and regulation of lipid transfer protein genes in cotton fiber, in: J.N. Jenkins, S. Saha (Eds.), Genetic Improvement of Cotton, Science Publishers, Inc., Enfield, NH, 2001, pp. 257–267.
[16] H.-C. Liu, R.G. Creech, J.N. Jenkins, D.-P. Ma, Cloning and promoter analysis of the cotton lipid transfer protein gene *Ltp*3, Biochim. Biophys. Acta 1487 (2000) 106–111.
[17] C.-Y. Hsu, R.G. Creech, J.N. Jenkins, D.-P. Ma, Analysis of promoter activity of cotton fiber lipid transfer protein gene *Ltp*6 in transgenic tobacco plants, Plant Sci. 143 (1999) 63–70.
[18] D.W. Hughes, G. Galau, Preparation of RNA from cotton leaves and pollen, Plant Mol. Biol. Reporter 6 (1988) 253–257.
[19] P.D. Siebert, A. Chenchik, D.E. Kellogg, K.A. Lukyanov, S.A. Lukyanov, An improved PCR method for walking in uncloned genomic DNA, Nucl. Acids Res. 23 (1995) 1087–1088.
[20] A. Feinberg, B. Vogelstein, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, Anal. Biochem. 132 (1983) 6–13.
[21] A.H. Paterson, C.L. Brubaker, J.F. Wendel, A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis, Plant Mol. Biol. Reporter 11 (1993) 122–127.
[22] A. Revizin, Gel electrophoresis assays for DNA–protein interaction, Biol. Tech. 7 (1987) 346–355.
[23] C.A. Beasley, In vitro culture of fertilized cotton ovules, Bioscience 21 (1971) 906–907.
[24] C.R. Walkerpeach, J. Velten, *Agrobacterium*-mediated gene transfer to plant cells: cointegrate and binary vector systems, in: S.B. Gelvin, R.A. Schilperoort (Eds.), Plant Molecular Biology Manual, Kluwer Academic Publishers, Belgium, 1994, pp. B1/1–B1/19.
[25] S.R. Gallagher, GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression, Academic Press, San Diego, CA, 1992.
[26] R.A. Jefferson, T.A. Kacanagh, M.W. Beva, GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J. 6 (1987) 3901–3907.
[27] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.
[28] A.E. Percival, R.J. Kohel, Distribution, collection, and evaluation of *Gossypium*, Adv. Agron. 44 (1990) 225–256.
[29] W. Xie, N.L. Trolinder, C.H. Haigler, Cool temperature effects on cotton fiber initiation and elongation clarified using in vitro cultures, Crop Sci. 33 (1993) 1258–1264.
[30] H. Jin, C. Martin, Multifunctionality and diversity within the plant MYB-gene family, Plant Mol. Biol. 41 (1999) 577–585.
[31] J.P. Tomkins, D.G. Peterson, T.J. Yang, D. Main, T.A. Wilkins, A.H. Paterson, R.A. Wing, Development of genomic resources for cotton (*Gossypium hirsutum* L.): BAC library construction, preliminary STC analysis, and identification of clones associated with fiber development, Mol. Breed. 8 (2001) 255–261.
[32] J.W. Grula, R.L. Hudspeth, S.L. Hobbs, D.M. Anderson, Organization, inheritance and expression of acetohydroxyacid synthase genes in the cotton allotetraploid *Gossypium hirsutum*, Plant Mol. Biol. (1995) 28.
[33] D.L. Ferguson, R.B. Turley, R.H. Kloth, Identification of a δ-TIP cDNA clone and determination of related A and D genome subfamilies in *Gossypium* species, Plant Mol. Biol. 34 (1997) 111–118.

[34] R.L. Small, J.F. Wendel, Copy number lability and evolutionary dynamics of the *Adh* gene family in diploid and tetraploid cotton (*Gossypium*), Genetics 155 (2000) 1913–1926.
[35] M.L. Cedroni, R.C. Cronn, K.L. Adams, T.A. Wilkins, J.F. Wendel, Evolution and expression of *MYB* genes in diploid and polyploid cotton, Plant Mol. Biol. 51 (2003) 313–325.
[36] K. Kosmidou-Dimitropoulou, Hormonal influences on fiber development, in: J.R. Mauney, J.Mc.D. Stweart (Eds.), Cotton Physiology, 1st ed. The Cotton Foundation, Memphis, TN, 1986, pp. 361–373.
[37] J.P. Rodgers, Cotton fruit development and abscission: variations in the levels of auxin, Trop. Agric. 58 (1981) 63–72.
[38] W.M. Gray, S. Kepinski, D. Rouse, O. Leyser, M. Estelle, Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins, Nature 414 (2001) 271–276.
[39] S. Kepinski, O. Leyser, Ubiquitination and auxin signaling: a degrading story, Plant Cell. 14 (2002) S81–S95.
[40] N. Ballas, L.-M. Wong, A. Theologis, Identification of the auxin-responsive element, AuxRE, in the primary indoleacetic acid-inducible gene, *PS-IAA*4/5, of pea (*Pisum sativum*), J. Mol. Biol. 233 (1993) 580–596.
[41] Z.-B. Liu, T. Ulmzsov, X. Shi, G. Hagen, T.J. Guilfoyle, The soybean *GH*3 promoter contains multiple auxin-inducible elements, Plant Cell. 6 (1994) 645–657.
[42] Y. Li, Z.-B. Liu, X. Shi, G. Hagen, T.J. Guilfoyle, Auxin-inducible elements in the soybean *SAUR* promoters, Plant Physiol. 106 (1994) 37–43.
[43] T. Ulmasov, Z.-B. Liu, G. Hagen, T.J. Guilfoyle, Composite structure of auxin response elements, Plant Cell. 7 (1995) 1611–1623.
[44] T. Guilfoyle, G. Hagen, T. Ulmasov, J. Murfett, How does auxin turn on genes, Plant Physiol. 118 (1998) 341–347.
[45] D. Jackson, F.A. Culianez-Macia, A.G. Prescott, K. Roberts, C. Martin, Expression patterns of *myb* genes from *Antirrhinum* flowers, Plant Cell. 3 (1991) 115–125.
[46] I. Romero, A. Fuertes, M.J. Benito, J.M. Malpica, A. Leyva, J. Paz-Ares, More than 80 *R2R*3-*MYB* regulatory genes in the genome of *Arabidopsis thaliana*, Plant J. 14 (1998) 273–284.
[47] J. Tanikawa, T. Yasukawa, M. Enari, K. Ogata, Y. Nishimura, S. Ishii, A. Sarai, Recognition of specific DNA sequences by the c-*myb* protooncogene product: role of three repeat units in the DNA-binding domain, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 9320–9324.
[48] T.A. Wilkins, F. Zhou, A novel cotton fiber R2R3-MYB transcription factor represses phenylpropanoid biosynthesis via a unique genetic mechanism, Plant Biol. Conf. Am. Soc. Plant Biologists (2002).
[49] H. Jin, E. Cominelli, P. Bailey, A. Parr, F. Mehrtens, J. Jones, C. Tonelli, B. Weisshaar, C. Martin, Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*, EMBO J. 19 (2000) 6150–6161.
[50] L. Tamagnone, A. Merida, A. Parr, S. Mackay, F.A. Culianez-Macia, K. Roberts, C. Martin, The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum* regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco, Plant Cell. 10 (1998) 135–154.
[51] L. Tamagnone, A. Merida, N. Stacey, K. Plaskitt, A. Parr, C.-F. Chang, D. Lynn, M. Dow, K. Roberts, C. Martin, Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants, Plant Cell. 10 (1998) 1801–1816.
[52] A. Mouradov, F. Cremer, G. Coupland, Control of flowering time: interacting pathways as a basis for diversity, Plant Cell. 14 (2002) S111–S130.
[53] R.N. Wilson, J.W. Heckman, C.R. Somerville, Gibberellin is required for flowering in *Arabidopsis thaliana* under shot days, Plant Physiol. 100 (1992) 403–408.
[54] S. Steiner-Lange, U.S. Unte, L. Eckstein, C. Yang, Z.A. Wilson, E. Schmelzer, K. Dekker, H. Saedler, Disruption of *Arabidopsis thaliana* MYB26 results in male sterility due to non-dehiscent anthers, Plant J. 34 (2003) 519–528.
[55] S. Fowler, M.F. Thomashow, *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway, Plant Cell. 14 (2002) 1675–1690.
[56] M.M. Lee, J. Schiefelbein, WEREWOLF: a MYB-related protein in *Arabidopsis*, is a position-dependent regulator of epidermal cell patterning, Cell 99 (1999) 473–483.
[57] J. Paz-Ares, D. Ghosal, U. Wienand, P.A. Peterson, H. Saedler, The regulatory *c*1 locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators, EMBO J. 6 (1987) 3553–3558.
[58] K.C. Cone, S.M. Cocciolone, F.A. Burr, B. Burr, Maize anthocyanin regulatory gene *pl* is a duplicate of *c*1 that functions in the plant, Plant Cell. 5 (1993) 1795–1805.